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MSX1 AND *TGFβ3* Gene Mutation and the Risk of Nonsyndromic Cleft Palate Only (NS CPO) Among Indonesian Patients

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Abstract : Non syndromic cleft palate only (NS CPO) is one of the most common congenital malformations that affect between 1 of 1000 - 2500 live births worldwide, which is considered as a genetically complex, multifactorial disease. Based on several association studies among the candidate genes with NS CPO, *MSX1* and *TGFβ3* genes emerged as the strong candidate genes in different populations with NS CPO. The objective of this study was to analyze the relationship between the mutations in *MSX1* gene (exon 1 C101G and exon 2 G817T) and *TGFβ3* gene (intron 3 T>A) and the risk of NS CPO in Indonesian patients.

This study was case control using samples from 22 NS CPO subjects and 43 control subjects. Venous blood samples were collected, then DNA was extracted and the segment of *MSX1* and *TGFβ3* genes were PCR-amplified. DNA sequencing from DNA fragments covering *MSX1* exon 1 C101G was performed by Sanger method. Digestion products containing *MSX1* exon 2 G817T and intron 3 T>A *TGFβ3* were evaluated. Statistical analysis used to determine significance differences from mutations frequency among both subjects was $\chi 2$. The odds ratio was used to determine a risk factor of NS CPO.

The study results showed that mutations in exon 1 C101G of *MSX1* gene were identified yet the differences in both subjects were not significant and also in *MSX1* gene exon 2 G817T as there was no mutation found in G817T. In *TGFβ3* intron 3 T>A, the frequency of A mutant allele was 35.7% in NS CPO and 28.8% in control. This difference was not significant statistically ($\chi 2$=0.748; p > 0,05), but the frequency of A mutant allele (odds ratio (OR) = 1.825; 95% CI = 0,635 - 5,245) and heterozygous mutant of TA genotype (OR = 1.941; 95% CI = 0,700 - 5,384) were associated with increased risk of NS CPO.

In conclusion, the mutations of *MSX1* gene in exon 1 C101G and exon 2 G817T are not considered to be a risk factor in Indonesian patients with NS CPO but *TGFβ3* intron 3 T>A can be considered to be the risk factor associated with NS CPO development in Indonesian patients.

Key words : nonsyndromic cleft palate only, gene mutation, *MSX1* gene, *TGFβ3* gene.

Introduction

Non syndromic cleft palate only (NS CPO) is one of the most common congenital malformations that affect between 1 of 1000 - 2500 live births worldwide, which is considered as genetically complex, multifactorial disease.[1]Genes and gene-environmental interactions have been believed to cause CPO [2]and genetic may play an important role as the etiology of CPO.Usually, the incidence of CL/P is higher than the incidence of cleft palate only (CPO), but the opposite result has been found in few studies.[1]Usually, the incidence of cleft lip only is lower than that of CPO. Birth prevalence of CPO and CL/P is generally higher in Asian populations compared to European populations.[3] The exact prevalence of CPO in Indonesia remains unknown.

Most CPO are of the non syndromic type that does not accompany syndromes, and this nonsyndromic type is known to affect as much as 70% of the whole CPO phenotypes.[2]Nonsyndromic oral clefts can be defined as complex traits, since they do not exhibit classic Mendelian recessive or dominant inheritance attributable to any single locus, but show strong familial aggregation and have a substantical genetic component.[2] NS CPO might be due to mutations in several different genes. Therefore, this study of CPO emphasizes on understanding the etiology of the nonsyndromic form.

The palate is phylogenetically an old structure. The beginning of the secondary palate is present in the most primitive reptiles. The development of the secondary palate in mammals has been an important stage in the evolution as maintenance of breathing, while the mouth is functioning in eating.[1]

Palatogenesis is a complex process that involves many genes.[3] Several loci and genes have been suggested as candidates of NS CPO. Based on several association studies among the candidate genes with NS CPO, Drosophila muscle segment homeobox homolog-1 (*MSX1)* and Transforming Growth Factor β3 (*TGFβ3)* genes emerged as the strong candidate genes in different populations with NS CPO, particularly in the region of *MSX1* gene exon 1 C101G, exon 2 G817T and *TGFβ3* gene (intron 3 T>A).[5,7] The *MSX1* gene maps to chromosome 4p16 [6] and play an important role in inductive epithelial-mesenchymal interactions leading to vertebrate organogenesis and especially palatal fusion.[6] The *TGFβ3* gene maps to chromosome 14q24[5], determines extracellular matrix protein accumulation by stimulating extracellular matrix protein neosynthesis, thereby inhibiting many enzymes which are responsible for extracelullar matrix protein degradation. Reduced activity of this gene due to mutation or polymorphism, may increase the risk for oral clefts.[7]The objective of this study was to analyze the relationship between the mutations in *MSX1* gene (exon 1 C101G and exon 2 G817T) and *TGFβ3* gene (intron 3 T>A) and the risk of NS CPO in Indonesian patients.

Experimental

Materials

This study was done by using samples from 22 NS CPO subjects and 43 control subjects and based on molecular epidemiology with case control study which is done in Molecular Biology Laboratory, *Unit penelitianKesehatan* (UPK) Faculty of Medicine/ HasanSadikin Hospital in Bandung.

Methods

DNA isolation. DNA was isolated from venous blood of each subjects using DNA isolation kit from Phamacia. Venous blood samples were collected with informed consent then DNA was extracted and the segment of *MSX1* and *TGFβ3* genes were polymerase chain reaction (PCR)-amplified.

PCR.The segment of *MSX1* gene to be ampilified can be seen in Figure 1.

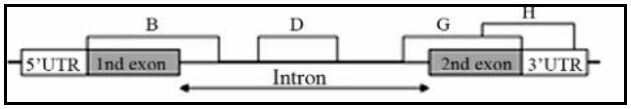

Figure1. B segment of the *MSX1* gene to be amplified [8]

PCR for *MSX1* gene exon 1 was performed by using the primers of: *forward* :5'-GGCTGCTGACAT GAC TTCTT-3' and *reverse* : 5'-AGGTCTGGAACCTTCTTCCTG-3' [8] which cover the exon 1 segment. Based on previous study done by Morkuniene et al (2006), there were mutations identified in exon 1 C101G *MSX1* gene in orofacial cleft including NS CPO patients.[10]

PCR for *TGFβ3* intron 3 T>A was performed by using the primers of forward : 5'-TATGTACATTTTCTT TGATCTCCCAGG-3' and reverse : 5'-TCAGCCTGGACAACATAGGGAGGACC-3'.[9]

PCR for *MSX1* G817T gene was performed by using the primers of forward: 5'- GGCTGATCATGCT CC AATGC-3' and reverse : 5' CAGGAAACAGCTATGACCCTGGAAGGGGCCAGAGGCTC-3'.[11]

DNA Sequencing. DNA sequencing covering *MSX1* gene exon 1 was performed by using dideoxy Sanger method. The sequencing process include the whole segment showed in figure 1. From the sequencing result from whole samples, all nucleotide in in those segment compared with normal nucleotide in gene bank by using sequence alignment programme from BioEdit.

Mutation analysis in *TGFβ3* T>A and *MSX1* 817 G>T genes was done by PCR- restriction fragment length polymorphism (RFLP) method by using the restriction enzymes of Arthrobacterprotophormiae (*Apo*I) dan Desulfovibriodesulfuricans (*Dde*I)[7,9,11], then digestion products containing *MSX1* exon 2 G817T and *TGFβ3* intron 3 T>A were evaluated.

RFLP for intron 3 *TGFβ3* T>A gene. PCR products from *TGFβ3* gene were digested with the specific restriction enzymes *Apo*1 at 37^0C.[9]

RFLP for *MSX1* G817T gene. The amplified PCR products of *MSX1* gene were digested with the specific restriction enzymes *Dde*I at 37^0C. The variant 817G>T does not create or disrupt the recognition site of *Dde*I because it designed as the mutagenesis primer to detect the mutation.[11]

All the digested PCR products were separated into channels on a 1.5% agarose gel containing ethidium bromide in an electrophoretic chamber. An ultraviolet transilluminator was used to see the specific band of base pairs of digested PCR products.

Statistical method. Statistical analysis which was used to determine significantly of differences from sequence variants frequency among NS CPO subject and control subject was χ^2. The odds ratio (OR) was used to determine a risk factor of NS CPO.

A P value of less than 0.05 was considered to be statistically significant. All of the analyses were performed using SPSS software, version 11.

Results

The initial PCR product of exon 1 *MSX1* gene showed DNA band segment with the size of 646 base pairs (bp). (Figure 2).

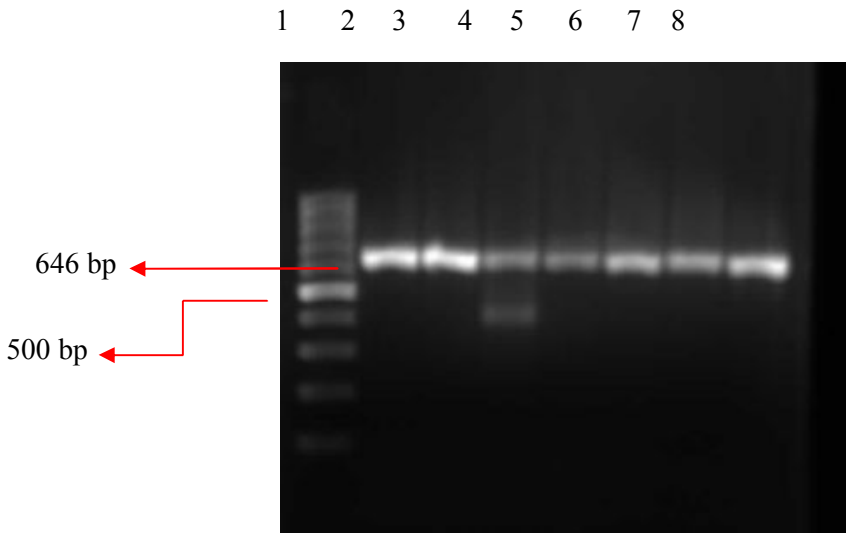

Figure 2. Initial PCR product of exon 1 *MSX1* gene Line 1. 100 bp ladder Line 2 – 8. Initial PCR product

The study results showed that mutation in exon 1 of *MSX1* gene were identified as C101G. The sequencing result from all subjects shows that there were gene mutations in the form of substituting C nucleotide into G nucleotide in base position of 101 (C101G)/rs36059701. In C101G it was found that there were the feature of normal CC genotype (Figure 3A), heterozygous mutant CG genotype (Figure 3B) in 2 samples of NS CPO and in 3 samples of normal subjects but no homozygous mutant of GG genotype found.

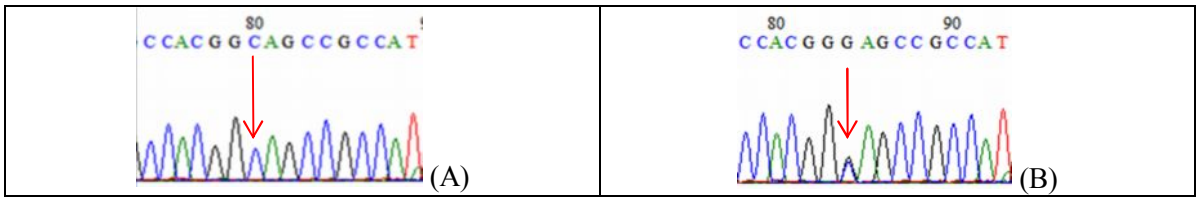

Figure 3. Sequencing result from *MSX1* gene exon 1 C101G. A. normal CC genotype of C101G, B. heterozygous mutant CG genotype of C101G

The initial PCR product of *MSX1* G817T gene showed DNA band segment with the size of 448 bp (Figure 4)

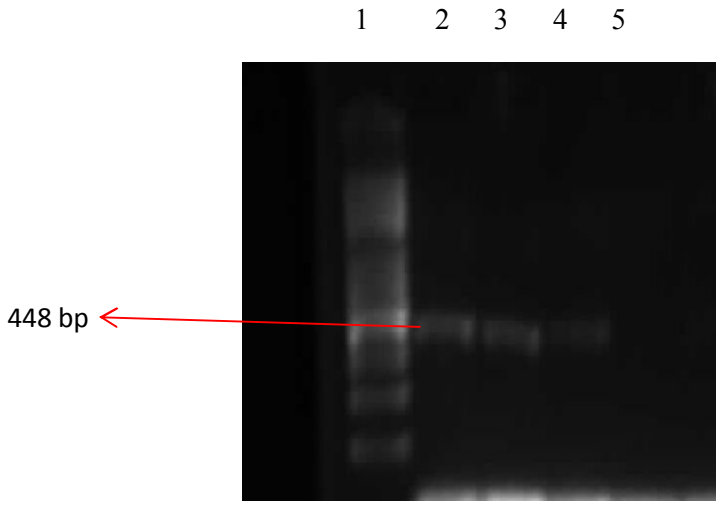

Figure 4. Initial product of *MSX1* geneLine 1. 100 bp ladderLine 2 – 5. Initial PCR product

Mutations of *MSX1* exon 2 G817T means that there is substituting G nucleotide into T nucleotide in base position of 817 (G817T). After obtaining the initial PCR products of *MSX1* gene, samples were then digested with the specific restriction enzyme *Dde*1. After digestion, the 484 bp products were completely digested with two restriction sites and three specific bands of 220, 150 and 39 bp (not shown) for homozygous normal of GG genotype feature as can be seen in this present study (Figure 5) and for heterozygous mutant of GT genotype feature it will show three specific bands of 220,181, 150 and 39 bp (not shown). For heterozygous mutant of GT genotype, the 220-bp PCR product of the mutant allele of the patient is further digested into 181- and 39 bp (not shown) products, present with the 220-bp product of the normal allele.[7,9]

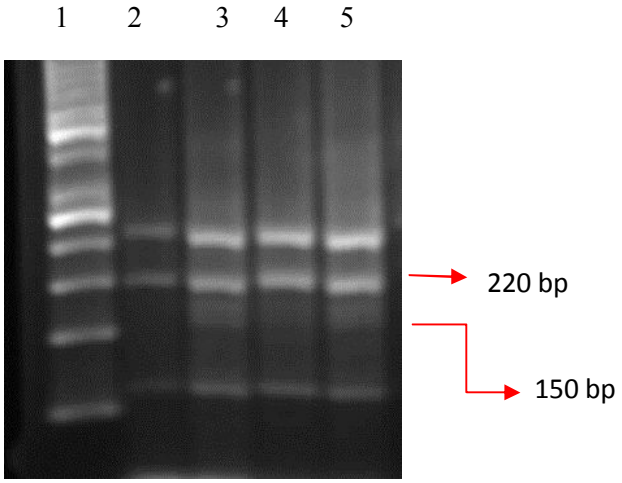

Figure 5.PCR products of *MSX*1 gene after restriction with *Dde*1 Line 1. 50 bp ladder Line 2-5. Homozygous normal of GG genotype

The initial PCR product of showed DNA band of *TGFβ3* gene(intron 3 T>A) segment and the size of this PCR product was 404 bp (Figure 6).

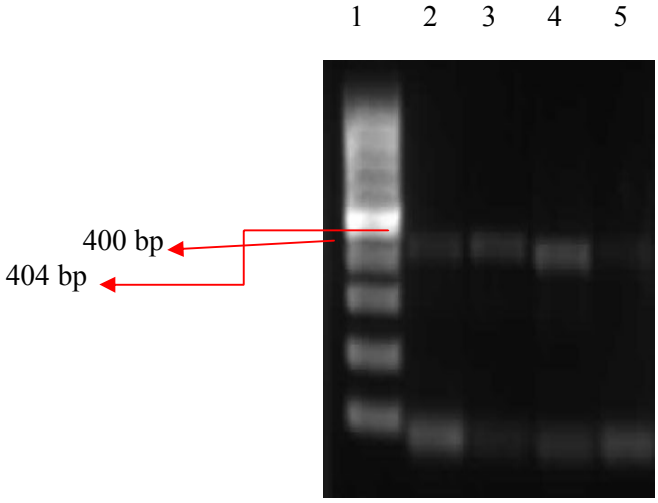

Figure 6. Initial PCR product of *TGFβ3*gene. Line 1. 100 bp ladder. Line 2 – 5. Initial PCR product

After obtaining the initial PCR products of *TGFβ3* gene (404 bp) then digested with the specific restriction enzyme *Apo*1, the 404 bp products were completely digested with two restriction sites and three specific bands of 214, 140 and 50 bp (not shown) was shown for homozygous mutant of AA genotype feature, and for heterozygous mutant of TA genotype feature, it will show three specific bands of 214, 140 ,50 bp and also band of 404 bp. The normal feature (homozygous normal of TT genotype) will show the bands of 404 bp and 50 bp. PCR products of *TGFβ3* gene after restriction with *Apo*1 can be seen in Figure 7.

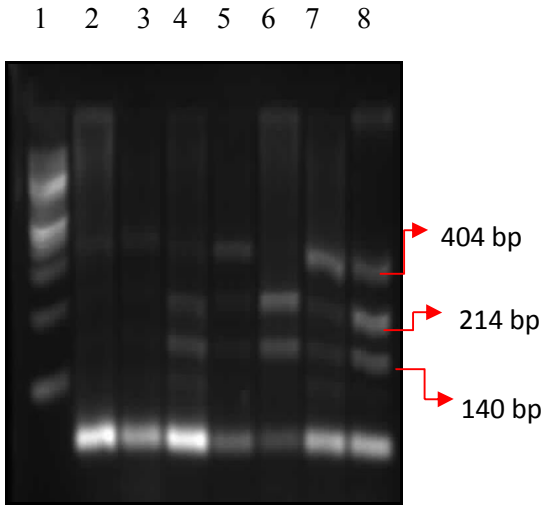

Figure 7. PCR products of *TGFβ3* gene after restriction with Apo1 . Line 1. 100 bp ladder Line 2. Homozygous normal of TT genotype Line 3, 4, 5, 7 and 8. Heterozygous mutant of TA genotype Line 6. Homozygous mutant of AA genotype

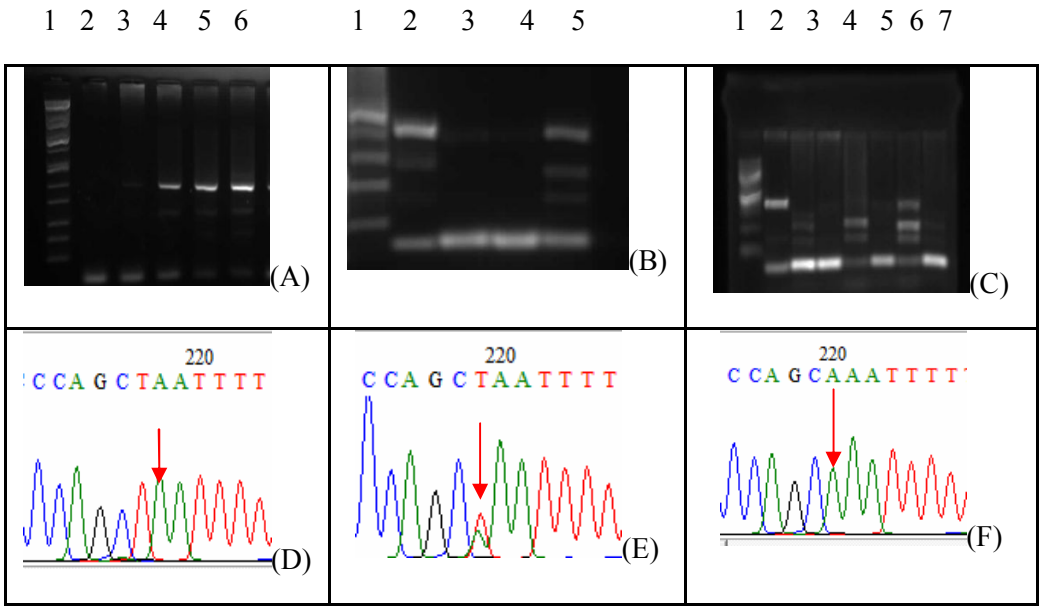

Figure 8. PCR products of *TGFβ3* gene after restriction with Apo1 and sequencing result. A and D (line 3-6 in A and the arrow in D) show homozygous normal of TT genotype. B and E (line 2 and 5 in B and the arrow in E) show heterozygous mutant of TA genotype. C and F (line 3 and 5 in A and the arrow in F) show Homozygous mutant of AA genotype

Statistical analysis of allelic frequency of A mutant allele and T normal allele from 22 NS CPO subjects and 43 normal subjects shown in Table 1. Statistical analysis of genotype frequency of homozygous normal TT genotype, heterozygous mutant TA genotype and homozygous mutant AA genotype from 22 NS CPO subjects and 43 normal subjects shown in Table 2.

In *TGFβ3* intron 3 T>A / rs2300607from this study, the frequency of A mutant allele was 35.7% in NS CPO subjects and 64,3% in control subjects. This difference was not so significant statistically ($\chi 2$=0.748; p > 0,05), but the frequency of A mutant allele (OR = 1.825; 95% CI = 0,635 - 5,245) and heterozygous mutant of TA genotype (OR = 1.941; 95% CI = 0,700 - 5,384) were associated with increased risk of NS CPO.

Discussion

Cleft palate is a common congenital malformation due to unknown etiological mechanisms. Normally the mouth is roofed by the hard and soft palate, which separate the oral cavity from the nasal cavities. The hard palate can be divided into the primary and the secondary palate. In humans the primary palate is anterior to and the secondary palate posterior to the foramen incisivum. The primary palate and the upper lip are formed from the medial nasal process by the end of the seventh developmental week.[12]At the same time, two palatal shelves are derived from the maxillar processes.[13] These are composed of mesenchymal cells surrounded by undifferentiated epithelial cells and the extracellular matrix. Unsulphatedglycosaminoglycans, collagen and other glycoproteins are the main components of the palatal extracellular matrix.[14]

The etiology and pathogenesis of cleft formation have been extensively studied but it is still poorly understood.[15,19] On the basis of mouse studies, cleft palate seems to be either a growth or a fusion failure of the secondary palate. In humans, some families with NS CPO show an autosomal dominant model of inheritance but, in most cases, the model of inheritance is not clearly mendelian. It has been widely accepted that the risk of recurrence is <2 % if one child already has CPO, <6 % if one parent has it and <15 % if one child and one parent have it. For a monozygous twin the risk is 50-60 %.[1] These facts clearly show that CPO has a strong genetic component. Numerous previous studies have suggested that many extrinsic factors might influence cleft formation. Thus, NS CPO is considered to be genetically complex, multifactorial diseases.[1] In this present study, we collected all samples without linked it into environmental factor, so it was purely only genetics point of view.

On the basis of mouse studies, cleft palate only seems to be either a growth or a fusion failure of the secondary palate.[1,4]The majority of such mutations have been found in the exon 1 of *MSX1* gene, suggesting the presence of hidden regulatory elements in this exon thus causing conservation of the sequence.[5]The *MSX1* gene is expressed at diverse sites of epithelial-mesenchymal interaction during vertebrate embryogenesis, and has been implicated in signalling processes between tissue layers then play an important role in inductive epithelial-mesenchymal interactions leading to palatal fusion and it has been proposed that the development of CPO is because of insufficient palatal mesenchyme.[6] Based on previous study done by Moukuniene et al (2006), there were mutations identified in exon 1 *MSX1* gene in NS CL/P patients in the form substituting nucleotide "C" into "G" of the 101 base pair (bp) region (C101G) resulted in substituting amino acid of alanine for glycine(A34G)[1] / rs36059701, so this mutation suppose to have a role to change transcription efficiency leads to some changes in quality, structure or protein function of this gene.[10,16]According to this study, mutation were identified at C101G in exon 1 of *MSX1* and among them 2 heterozygous mutant of CG genotypes were found in CPO subjects and 3 heterozygous mutant of CG genotypes were found in normal subjects, means that this difference wasn't significant statistically and the risk factor can not be determined.

MSX1 G817T located in exon 2. Based on the previous study done by VP Singh & D. Ramu*MSX1* G817T gene mutation has a role in the etiology of NS CL/P in South India and Thailand population. This study, however, is contrary to a study carried out by Lidral et al. in Philippines population, which states that this variant does not play a significant role in aetiology of NS CPO. These differences could be attributed to genetic differences in populations.[7,10]In all subjects of this study, it was observed that there was no significant association with the *MSX1* G817T gene mutation.

TGFβ3 gene is expressed in the medial edge epithelium cells of palatal shelves and thought to contribute to palatal shelf elongation and fusions. Knockout mice studies with TGFβ3 gene deletion have shown development of CPO.[17,18]Based on previous study done by Singh VP et al in South Indian population, C. Lidral et al in South American population and J. Suazo et al in Chilean population indicate that there is strong association between the presence of *TGFβ3* (intron 3 T>A) / rs2300607mutations with the incidence of non syndromic cleft lip and palate but these studies are contrary to a study done by Andrew C. Lidral et al in Philippines population which may be due to genetic differences in that population. The results showed that the frequency of A mutant allele was 35.7% in NS CPO subjects and 28.8% in control subjects. This difference was not so significant statistically ($\chi 2$=0.748; p > 0,05), but the frequency of A mutant allele (odds ratio (OR) = 1.825; 95% CI = 0,635 - 5,245) and TA genotype (OR = 1.941; 95% CI = 0,700 - 5,384) were associated with increased risk of NS CPO as it means that the presence A mutant alelle has a risk 1.825 times for the NS CPO and the presence of heterozygous mutant of TA genotype has a risk 1.941 times for the NS CPO followed by the presence of homozygous mutant of AA genotype which has a risk 1.655 for the NS CPO.

Conclusion

The mutations of *MSX1* gene in exon 1 and exon 2 G817T are not considered as a risk factor in Indonesian patients with NS CPO, but *TGFβ3* intron 3 T>A can be considered to be the risk factor associated with NS CPO development in Indonesian patients.

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